Virology **292,** 78–86 (2002)
doi:10.1006/viro.2001.1230, available online at http://www.idealibrary.com on IDEAL®

Restriction of a Sindbis Virus Mutant in BHK Cells and Relief of the Restriction by the Addition of Adenosine

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Received August 1, 2001; returned to author for revision August 27, 2001; accepted October 11, 2001

SV_{PZF} is a mutant of Sindbis virus (SV) which we selected on the basis of its ability to replicate in mosquito cells treated with pyrazofurin (PZF), a drug which inhibits pyrimidine nucleotide biosynthesis (Lin *et al.,* 2000, *Virology* 272, 61–71). Three mutations, A6627U, A7543U, and C7593A, were identified in the nsP4 (the viral RNA polymerase) coding region, which were required for the PZF-resistant phenotype. We report here that SV_{PZF} has a second phenotype. Its replication in BHK cells is severely restricted; yields of SV_{PZF} from BHK cells are 100- to 1000-fold lower than the yields of standard SV (SV_{STD}). However, addition of adenosine to the SV_{PZF}-infected cultures completely relieves this restriction and results in yields comparable to those observed with SV_{STD}. Adenosine has no effect on the yield of SV_{STD} from BHK cells. Synthesis of the viral structural proteins is markedly depressed in SV_{PZF}-infected BHK cells, as is synthesis of the viral subgenomic (SG) RNA from which these proteins are translated. In contrast, normal amounts of genomic RNA are made. Experiments with mutagenized viruses indicated that the SV_{PZF} mutation, C7593A, by itself, was sufficient to produce the restriction phenotype. However, this mutation not only changes Pro 609 of nsP4 to Thr, it also changes the nucleotide at the −5 position of the SG promoter. To evaluate the relative contributions of the change in nsP4 and the change in the SG promoter to the restriction phenotype, we made use of double SG viruses, in which nsP4 and the promoter for the SG RNA which encodes the structural proteins can be changed independent of each other. Our results indicated that both the change in nsP4 and the change in the SG promoter were required to produce the full restriction phenotype. We suggest that the changes in nsP4 and the SG promoter destabilize the RNA initiation complex assembled at the SG promoter and that since ATP is the initiating nucleotide in the SG RNA transcript, the increased level of ATP resulting from the addition of adenosine is able to compensate for this destabilization and restore the synthesis of SG RNA to normal levels.

INTRODUCTION

Sindbis virus (SV) is the prototype virus of the family *Togaviridae,* genus *Alphavirus.* The 11,703 nt-long, positive-sense, single-stranded RNA genome is capped at its 5′ end and polyadenylated at its 3′ end. The 5′ two-thirds of the genomic RNA encodes four nonstructural proteins. The three structural proteins (the capsid protein, C, and the two envelope proteins, E1 and E2) are translated from a subgenomic RNA, which is transcribed from an internal subgenomic (SG) promoter on the full-length, negative-strand RNA (reviewed in Strauss and Strauss, 1994). The minimal sequence which has promoter activity extends from nt −19 to nt +5, where the SG transcript begins at nt +1 (Hertz and Huang, 1992; Levis *et al.,* 1990).

The four nonstructural proteins, nsP1, nsP2, nsP3, and nsP4, are derived by the processing of two polyproteins, P123 and P1234. The synthesis of negative-strand and positive-strand genomic RNA and the transcription of the subgenomic RNA are regulated temporally by the processing of the nonstructural polyproteins (Lemm *et al.,* 1994, 1998; Sawicki and Sawicki, 1998). Thus, once the cleavage between the nsP2 and the nsP3 proteins takes place, the synthesis of negative-strand RNA ceases and the synthesis of the positive-strand genomic and subgenomic RNA becomes more efficient. As to the functions of the nonstructural proteins, nsP1 has both RNA methyltransferase (Mi *et al.,* 1989; Mi and Stollar, 1990) and RNA guanylyltransferase activities (Ahola and Kääriäinen, 1995; Scheidel *et al.,* 1987). Thus, nsP1 is involved in the modification of the 5′ ends of the positive-strand genomic and subgenomic RNAs. nsP2 is a multifunctional protein. Its N-terminal domain has an RNA helicase activity, which may unwind the replicative RNAs during replication (Gomez *et al.,* 1999). Recently, an RNA triphosphatase activity has also been associated with this domain; thus nsP2 appears to also be involved in the capping of the viral RNA along with nsP1 (Vasiljeva *et al.,* 2000). The C-terminal domain of nsP2 is a papain-like protease, which is responsible for the processing of the

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0042-6822/02 $35.00

nonstructural polyproteins P123 and P1234 (Hardy and Strauss, 1989). nsP3 is the only one of the nonstructural proteins which is phosphorylated (Peranen *et al.,* 1988; Li *et al.,* 1990) but its biochemical function is still unknown. nsP4 has been identified as the viral RNA polymerase since it contains the consensus GDD sequence found in nearly all RNA-dependent RNA polymerases (RDRP) (Kamer and Argos, 1984). Experiments with a ts mutant, ts-6, in which the mutation lies in the nsP4-coding region, also point to nsP4 as the viral RNA polymerase (Barton *et al.,* 1988).

Alphaviruses have an extremely broad host range. In their natural ecosystem, they are maintained by a mandatory vertebrate–mosquito–vertebrate transmission cycle. In order to successfully replicate in such different hosts, the viral proteins involved in virus assembly and viral RNA synthesis must be able to function efficiently in host cells that are widely separated phylogenetically. In the laboratory, however, it is possible to obtain host range mutants of Sindbis virus which replicate well in one host cell but are restricted in a different cell type. Thus, viral mutants with mutations in the E2 coding sequence have been described which replicate well in mosquito cells but are restricted in chicken embryonic fibroblasts (CEF) and baby hamster kidney (BHK) cells (Li *et al.,* 1999; Durbin and Stollar, 1984). Another Sindbis virus mutant has been described that does not cleave PE2 to E2 and is restricted in mosquito cells but not in vertebrate cells (Boehme *et al.,* 2000).

The structure or amount of nsP4 also appears to influence the host range of Sindbis virus. Three different mutations, which mapped to the N-terminus of nsP4, when introduced into a wild-type background led to a restriction of replication in mosquito cells but not in BHK cells (Kowal and Stollar, 1981; Lemm *et al.,* 1990).

Finally, for reasons that are not clear, the sequence of the Sindbis virus subgenomic promoter can also influence the host range. One possible explanation may relate to the participation of host factors in the assembly of the RNA synthesizing complex at the promoter for the SG RNA. In their studies on the evolution of the SG promoter sequences from nt −13 to nt −9, Hertz and Huang (1995a) identified within this region (nt 7587–7591 of the SV genome) three sequences with two, three, or four changes from the wild-type sequence, which reduced the yield of virus from mosquito cells much more severely than from BHK cells.

Recently, we reported the selection in mosquito cells of a mutant Sindbis virus, SV_{PZF}, which is resistant to pyrazofurin (PZF), a compound that inhibits orotic acid monophosphate decarboxylase and thus interferes with the synthesis of UTP and CTP (Lin *et al.,* 2000). Four mutations, one in the nsP3 and three in the nsP4 coding region, were identified. The mutation in the nsP3 coding region changes the opal stop codon to Cys. However, only the three mutations in the nsP4 coding region were required to produce the PZF-resistant phenotype. On the basis of a molecular model of nsP4, we localized the N-terminal amino acid change to the fingers domain of nsP4, i.e., the RNA polymerase, and the other two to the thumb domain. We suggested that these three amino acid changes in nsP4 altered the geometry of the NTP binding pocket, increasing the binding affinity of the mutant viral RNA polymerase for UTP and CTP and enabling SV_{PZF} to replicate in cells with low levels of UTP and CTP.

We now report that, in addition to its PZF-resistant phenotype, SV_{PZF} has a host range phenotype. Although it replicates well in mosquito cells (Lin *et al.,* 2000), it is severely restricted in BHK cells. Only one of the SV_{PZF} mutations is required to produce the restriction phenotype, that at nt 7593. However, this mutation not only changes Pro 609 of nsP4 to Thr, but also changes the C at the −5 position of the subgenomic promoter to an A. Making use of double subgenomic Sindbis viruses, we showed that both changes were necessary to produce the strong restriction phenotype.

TABLE 1

The Replication of SV_{PZF} Is Restricted in BHK Cells: Adenosine Relieves the Restriction

		SV_{STD}	SV_{PZF}
−Actinomycin D 0.5 μg/ml	No treatment	7.6×10^9	4.7×10^6
	300 μM Adenosine	1.6×10^9	1.1×10^9
	300 μM Guanosine	9.1×10^9	6.0×10^7
+Actinomycin D 0.5 μg/ml	No treatment	4.6×10^9	3.8×10^6
	300 μM Adenosine	4.3×10^9	2.1×10^9

Note. Cells were infected and maintained as described under Materials and Methods. Actinomycin D, adenosine, and guanosine were added as indicated. Medium was harvested at 20 h postinfection and assayed for infectious virus by plaque assay on CEF. This experiment was done three times. The results in all three experiments were similar.

RESULTS

SV_{PZF} is restricted in BHK cells

Table 1 illustrates the restriction of SV_{PZF} in BHK cells. The yield of SV_{PZF} from BHK cells was approximately 1000-fold lower than that of standard Sindbis virus (SV_{STD}). However, the addition of adenosine (300 μM final concentration) to the infected BHK cells increased the yield of SV_{PZF} to a level comparable to that of SV_{STD} and thus completely relieved the restriction of SV_{PZF} in BHK cells. The same concentration of guanosine also increased the yield of SV_{PZF} from BHK cells, but only 10-fold. Addition of actinomycin D (0.5 μg/ml) had no effect on either the restriction phenotype of SV_{PZF} or the relieving effect of adenosine. Adenosine had no effect on the yield of SV_{STD}.

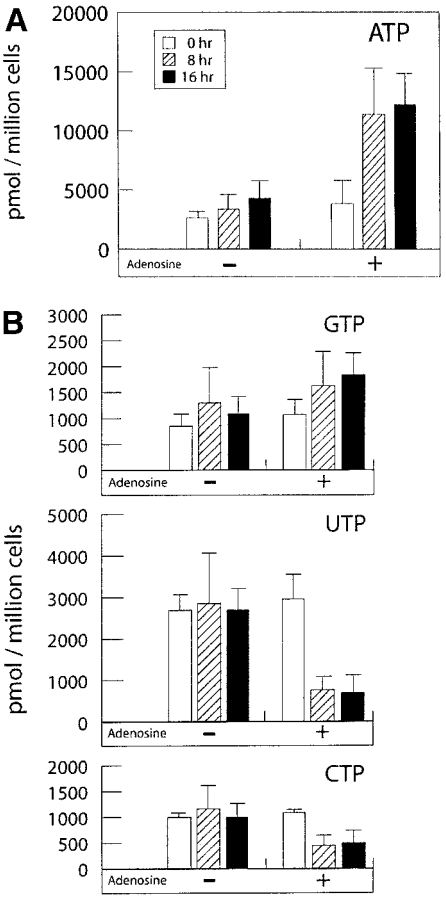


FIG. 1. Effect of adding adenosine to BHK cells on the levels of (A) ATP and (B) GTP, UTP, and CTP. BHK cells were mock-infected and treated with adenosine as indicated. Ribonucleotides were extracted at 0, 8, and 16 h and analyzed as described under Materials and Methods.

SV_{PZF} showed a mild restriction in HeLa cells; its yield was about 10-fold lower than that of SV_{STD}. Addition of adenosine to SV_{PZF}-infected cells abolished this difference. In CEF no significant restriction was observed.

To help clarify the possible mechanisms whereby adenosine relieves the restriction of SV_{PZF} in BHK cells, we measured the levels of ribonucleotides in BHK cells, which had been treated with 300 μM adenosine or had been untreated. Figure 1A shows that, by 8 h after treatment, the level of intracellular ATP was increased approximately three- to fourfold (relative to the zero time or to untreated cells). There was a small increase in the level of GTP, the significance of which is uncertain (Fig. 1B). In contrast, the levels of UTP and CTP were decreased about five- and twofold, respectively. The reductions in levels of UTP and CTP following addition of adenosine to BHK cells recall a similar effect of adenosine on the UTP and CTP pools in mosquito cells (Stollar and Malinoski, 1981).

To determine at which stage the replication of SV_{PZF} was restricted in BHK cells, we first examined the synthesis of viral proteins by labeling infected cells with [^{35}S]methionine. Figure 2 shows that the synthesis of the

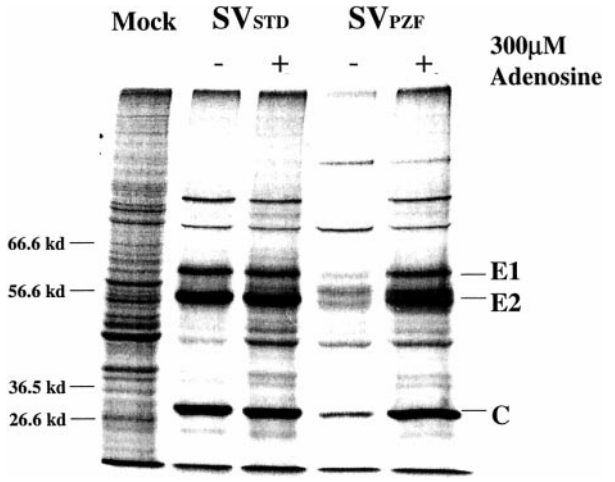


FIG. 2. Viral protein synthesis in BHK cells infected with SV_{STD} or SV_{PZF}. Cells were infected and maintained as described under Materials and Methods. Actinomycin D (0.5 μg/ml) was added to all cultures, and adenosine was added as shown. At 7 h after infection, the medium was replaced with methionine-free medium, and the cells were labeled with ^{35}S-labeled protein labeling mix (10 μCi/35-mm plate) from 7 to 9 h after infection. Lysates corresponding to 0.5×10^6 cells were analyzed using 10% SDS–PAGE with a stacking gel. The gel was dried and exposed to X-ray film.

viral structural proteins was inhibited in the SV_{PZF}-infected BHK cells. The addition of adenosine, however, increased the synthesis of these viral proteins to a level similar to that seen in the SV_{STD}-infected BHK cells.

To determine whether the reduced viral protein synthesis in SV_{PZF}-infected BHK cells was a result of decreased synthesis of viral RNA, specifically of the SG RNA, we compared the synthesis of viral RNA in SV_{STD}- and SV_{PZF}-infected cells by labeling cells with ^{32}P-labeled inorganic phosphate. As seen in Fig. 3, there were sev-

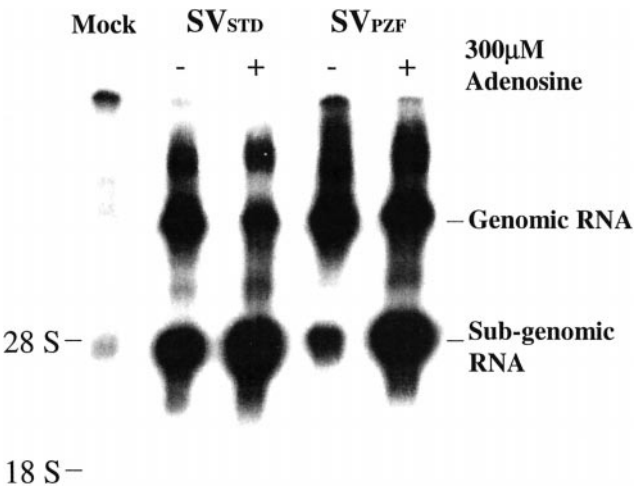


FIG. 3. Viral RNA synthesis in BHK cells infected with SV_{STD} or SV_{PZF}. Viral RNA, labeled with [^{32}P]orthophosphate from 3 to 8 h after infection, was extracted and analyzed as described under Materials and Methods.

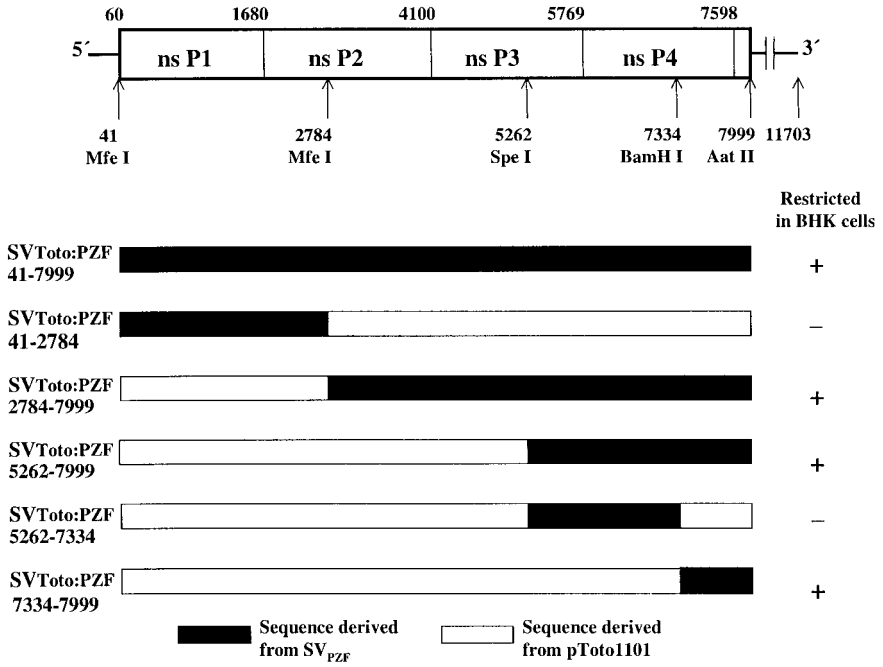


FIG. 4. Restriction of chimeric viruses containing various sequences derived from SV_{PZF} genome. BHK cells were infected (10 PFU/cell) with the chimeric viruses shown above and maintained as described under Materials and Methods. Adenosine (500 μM) was added as indicated. Medium was harvested at 20 h postinfection and assayed for infectious virus by plaque assay on CEF. A plus sign indicates that the viral yield in the absence of adenosine was at least 100-fold lower than in the presence of adenosine, i.e., that the virus was restricted in BHK cells.

eral significant findings: (1) overall viral RNA synthesis was not greatly reduced, relative to SV_{STD}-infected cells; (2) the amount of viral SG RNA made in SV_{PZF}-infected cells was much less than in the SV_{STD}-infected cells; and (3) the SV_{PZF}-infected cells, in contrast to the SV_{STD}-infected cells, made much less SG RNA than genomic RNA. When adenosine was added to the SV_{PZF}-infected cultures, it dramatically increased the synthesis of SG RNA. Adenosine also increased the synthesis of SG RNA in SV_{STD}-infected cells, apparently at the expense of genomic RNA. Using the Kodak Electrophoresis Documentation and Analysis System 120 we have evaluated these data more quantitatively. In the SV_{STD}-infected cells in the absence of adenosine, the ratio of SG RNA to genomic RNA synthesized was 1.6; when adenosine was added, this ratio increased to 5.3. Thus, in both instances more SG RNA was made than genomic RNA. In the SV_{PZF}-infected cells, in the absence of adenosine the ratio of SG to genomic RNA synthesized was only 0.3, but when adenosine was added the ratio increased to 2.4. These results suggest that the restriction of SV_{PZF} in BHK cells may result from a decreased synthesis of SG RNA.

Localization and identification of the mutation(s) responsible for restriction of SV_{PZF} in BHK cells

To determine which region of the SV_{PZF} genome is responsible for its restriction in BHK cells, we examined six different chimeric viruses containing SV_{PZF} cDNA sequences in an SV_{TOTO} background (Lin *et al.,* 2000), to see which were restricted in BHK cells. Four of the chimeric viruses were restricted (Fig. 4). These were SV_{TOTO}:PZF:41-7999, SV_{TOTO}:PZF:2784-7999, SV_{TOTO}:PZF:5262-7999, and SV_{TOTO}:PZF:7334-7999. In contrast to SV_{STD}, all four viruses showed at least a 100-fold or higher increase in titer when infected BHK cells were treated with adenosine (data not shown). These results indicate that the SV_{PZF} sequence from nt 7334 to nt 7999 is sufficient to cause the restriction phenotype. Two of the SV_{PZF} mutations (A7543U, C7593A), which we described previously (Lin *et al.,* 2000), lie within this region.

In our earlier work (Lin *et al.,* 2000), we had generated viruses which contained all possible combinations of the SV_{PZF} mutations in an SV_{TOTO} background. These mutants were used to determine which of the SV_{PZF} mutations were required for the restriction phenotype. Since only two of these mutations, A7543U and C7593A, lie within the nt 7334–7999 region, we expected that either one or both would suffice to cause the restriction phenotype. For convenience we refer to the mutations at nt 5750, 6627, 7543, and 7593 as mutations 1, 2, 3, and 4, respectively.

Table 2 shows that when mutations 1, 2, 3, or 4 were introduced one at a time, only mutation 4 produced the restriction phenotype; when the mutations were introduced as pairs, only 2/4 and 3/4 gave the restriction phenotype. Interestingly, the 1/4 pair consistently showed little restriction in BHK cells, suggesting that mutation 1 may in some way diminish the effect of mutation 4.

TABLE 2

BHK Restriction Phenotypes of Viruses with Various SV_{PZF} Mutations

Virus	Adenosine (500 μM) − (a)	Adenosine (500 μM) + (b)	b/a	Restriction
SV_{STD}	1.0×10^9	6.2×10^8	0.6	−
SV_{PZF}	4.9×10^6	5.7×10^8	116	+
SV_{Mut1}	9.4×10^8	8.7×10^8	0.9	−
SV_{Mut2}	3.8×10^8	5.8×10^8	1.5	−
SV_{Mut3}	6.2×10^8	1.3×10^9	2.1	−
SV_{Mut4}	2.4×10^7	1.4×10^9	58	+
$SV_{Mut1,2}$	3.4×10^8	6.8×10^8	2.0	−
$SV_{Mut1,3}$	4.0×10^8	8.5×10^8	2.1	−
$SV_{Mut1,4}$	6.1×10^8	1.2×10^9	2.0	−
$SV_{Mut2,3}$	1.3×10^8	6.8×10^8	5.5	−
$SV_{Mut2,4}$	4.7×10^6	2.5×10^8	53	+
$SV_{Mut3,4}$	1.3×10^7	6.2×10^8	48	+
$SV_{Mut1,2,3}$	2.1×10^8	9.3×10^8	4.4	−
$SV_{Mut1,2,4}$	1.3×10^7	6.5×10^8	50	+
$SV_{Mut1,3,4}$	2.1×10^7	7.7×10^8	37	+
$SV_{Mut2,3,4}$	2.3×10^6	3.5×10^8	152	+
$SV_{Mut1,2,3,4}$	6.2×10^6	3.4×10^8	55	+

Note. BHK cells were infected and maintained as described under Materials and Methods with or without 500 μM adenosine. Medium was harvested at 20 h postinfection and assayed for infectious virus by plaque assay on CEF. A plus sign indicates that the virus was restricted. In this experiment, a difference between the virus yield in the absence and in the presence of adenosine of close to 40-fold or greater was taken as evidence of restriction. This experiment was done three times. Similar results were obtained in all three experiments.

[a] Mutation 1, A5750U; mutation 2, A6627U; mutation 3, A7543U; mutation 4, C7593A.

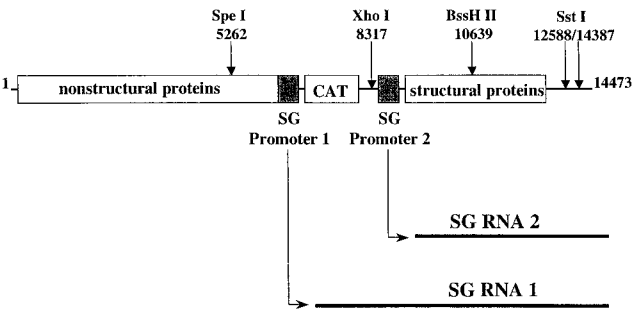
Combinations of three mutations led to restriction in BHK cells only if mutation 4 were present. The combinations 1/2/4 and 1/3/4 showed the restriction phenotype but not to the same degree as the combination 2/3/4. In these experiments, mutations 1/2/3/4 also showed a lower degree of restriction than the combination 2/3/4.

From these results, we conclude that the SV_{PZF} mutation 4 alone is sufficient to cause restriction in BHK cells but that mutations 2 and 3 enhance the restriction. Mutation 1, on the other hand, appears to counteract the effect of mutation 4.

The restriction of SV_{PZF} in BHK cells requires both the mutated SG promoter and the amino acid change in nsP4 at position 609

The coding region for the C-terminus of nsP4 of Sindbis virus overlaps with the SG promoter, i.e., the promoter from which the transcription of SG RNA is initiated (Strauss *et al.,* 1984). Thus the SV_{PZF} mutation at nt 7593, which was required and sufficient for the restriction in BHK cells, not only results in an amino acid change (Pro to Thr) at position 609 of nsP4 but also causes a C to A transversion at the −5 position of the minimal SG promoter (Levis *et al.,* 1990). Accordingly, the reduction of viral SG RNA synthesis in SV_{PZF}-infected BHK cells (Fig. 3) could be mediated by the mutated promoter, the altered RNA polymerase, or an interaction between the two. To distinguish among these possibilities, we made use of a double subgenomic Sindbis virus. This virus was obtained by transfecting cells with RNA transcribed from the plasmid, pTCS (Hertz and Huang, 1995a,b); see Fig. 5. In this double SG virus, SV_{TCS}, the SG promoter 1, as in the normal SV, overlaps with the coding sequence for the C-terminus of nsP4, but plays no role in the expression of the viral genes encoding the structural proteins. SG promoter 2, which is upstream of the genes for the structural proteins C, E2, and E1, controls the expression of these genes, but does not overlap with the nsP4 coding sequence. Thus, with SV_{TCS}, it is possible to mutate the SG promoter upstream of the genes for the structural proteins without altering nsP4 and, conversely, to mutate the nsP4 coding region, i.e., to introduce the change at residue 609 of nsP4, without altering the SG promoter from which the RNA encoding the structural proteins is transcribed.

We therefore introduced SV_{PZF} mutation 4 into SG promoter 1 (thereby changing the amino acid at position 609 of nsP4), into SG promoter 2, or into both promoters of SV_{TCS}. The three mutagenized double SG viruses were then tested, along with the parental SV_{TCS}, for the restriction phenotype in BHK cells. SV_{TCS} encodes a wild-type nsP4 and wild-type SG promoters for SG RNA 1 and RNA 2. SV_{mSG1} carries the C to A mutation at nt 7593, which results in a mutant nsP4, as well as a mutant SG promoter 1 for SG RNA 1, but it retains the wild-type promoter for SG RNA 2. SV_{mSG2} encodes a wild-type nsp4 but has the SV_{PZF} mutation 4 (C8422A) at the −5 position of SG promoter 2. $SV_{mSG1SG2}$ contains mutations in both promoters and therefore generates a mutant form of nsP4, along with mutations in both SG promoters.



Linear map of pTCS

FIG. 5. Linear map of pTCS. The nucleotide numbering begins with the first base of the SV RNA, followed by the vector sequence. Infectious Sindbis virus containing double subgenomic promoters was generated by linearizing the plasmids and then transfecting cells with RNA transcripts of the plasmid.

TABLE 3

Restriction of SV with dSG Promoters

		SV_{STD}	SV_{PZF}	SV_{TCS}	SV_{mSG1}	SV_{mSG2}	$SV_{mSG1SG2}$
Adenosine	0 μM (a)	3.1×10^9	6.0×10^6	7.0×10^8	9.0×10^7	2.9×10^7	5.0×10^6
	300 μM (b)	4.9×10^9	6.1×10^8	5.1×10^9	2.0×10^9	8.0×10^8	1.8×10^9
	b/a	1.6	102	7.3	22	28	360

Note. Cells were infected and maintained as described under Materials and Methods. Cultures were treated with actinomycin D (0.5 μg/ml) and 300 μM adenosine as indicated. Medium was harvested at 20 h postinfection and assayed for infectious virus by plaque assay on CEF. SV_{TCS} has wild-type SG1 and SG2 promoters. SV_{mSG1} has a C to A mutation at the −5 position of the SG1 promoter. SV_{mSG2} has a C to A mutation at the −5 position of SG promoter 2. $SV_{mSG1SG2}$ has C to A mutations at the −5 position of both SG promoters. This experiment was done four times. Similar results were obtained each time.

Table 3 shows that SV_{TCS} (the double subgenomic virus without any mutations) replicated well both in the absence and in the presence of adenosine. In contrast, $SV_{mSG1SG2}$ was severely restricted in BHK cells, and just as is the case with SV_{PZF}, the restriction was relieved by the addition of adenosine. However, SV_{mSG1}, which encodes a mutant nsP4 but does not change SG promoter 2, showed only a modest restriction. A similar low degree of restriction, again relieved by adenosine, was observed for SV_{mSG2}, which encodes a wild-type nsP4, but has a mutant SG promoter 2. As with $SV_{mSG1SG2}$, the addition of adenosine (300 μM) increased the yield of both SV_{mSG1} and SV_{mSG2}. We conclude from these results that the full restriction phenotype in BHK cells, as displayed by SV_{PZF}, requires both the amino acid change in nsP4 and the mutation in SG promoter 2.

We next proceeded to measure the synthesis of viral RNA and viral proteins in cells infected with the different double SG viruses. Figure 6 shows that mutation of SG promoter 2, but not of SG promoter 1, led to decreased synthesis, relative to SV_{TCS}-infected cells, of all the viral structural proteins. Mutations in both promoters also resulted in an inhibition in the synthesis of these proteins. Mutation of SG promoter 1, i.e., changing amino acid 609 of nsP4, had no effect on the synthesis of the viral structural proteins. The addition of adenosine (300 μM) increased the synthesis of the viral structural proteins in the SV_{mSG2}- and $SV_{mSG1SG2}$-infected BHK cells to a level comparable to that observed in the SV_{TCS}- and SV_{mSG1}-infected cells. Adenosine also slightly increased the synthesis of the viral structural proteins in the SV_{TCS}- and SV_{mSG1}-infected cells. These results clearly indicate that the amino acid change at position 609 of nsP4 by itself does not reduce synthesis of the viral structural proteins.

Next we examined the synthesis of viral SG RNA in cells infected with the mutated double SG viruses. Figure 7 shows that the synthesis of SG RNA 1 (transcribed from the SG promoter 1 of SV_{TCS}) was decreased in SV_{mSG1}-infected cells, that the synthesis of SG RNA 2 (transcribed from the SG promoter 2 of SV_{TCS}) was decreased in SV_{mSG2}-infected BHK cells, and that the synthesis of both SG RNA 1 and RNA 2 was decreased in $SV_{mSG1SG2}$-infected BHK cells. In each case, the addition of adenosine (300 μM) restored the synthesis of the SG RNA to

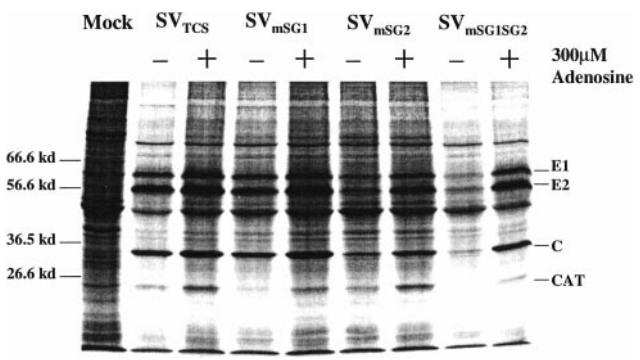


FIG. 6. Viral protein synthesis in BHK cells infected with double subgenomic Sindbis viruses with mutated SG promoters. Cells were infected with the various double SG viruses as indicated. The procedure was as described for Fig. 2, except that cells were incubated with methionine-free medium from 8 to 9 h postinfection and then labeled with ^{35}S-labeled protein labeling mix (100 μCi/35-mm plate) from 9 to 10 h after infection.

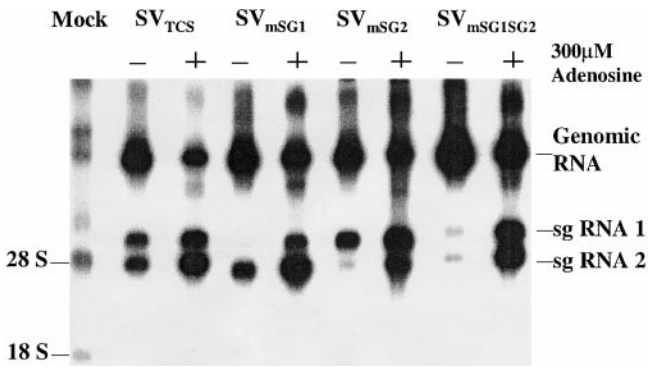


FIG. 7. Viral RNA synthesis in BHK cells infected with double subgenomic Sindbis viruses with mutated SG promoters. Cells infected and treated as described under Materials and Methods were incubated with ^{32}P-labeled orthophosphate (100 μCi/35-mm plate) from 3 to 8 h after infection. The total RNA was isolated with TriZol reagent, denatured in buffer containing formaldehyde and de-ionized formamide, and then resolved in 1% formaldehyde–agarose gels. The gel was dried and exposed to X-ray film.

the level seen with SV_{TCS}. Thus, as was the case with the reduced synthesis of the viral structural proteins, the reduced synthesis of viral SG RNA 2 (the RNA which encodes the structural proteins) required only the mutation in the promoter for this RNA (SG promoter 2) but not the alteration in nsP4.

DISCUSSION

The SV_{PZF} mutant has features which make it of special interest. To our knowledge, it is the only example of a viral mutant which has been selected on the basis of its ability to grow in cells with decreased levels of rNTPs. The demonstration that the host restriction phenotype can be completely relieved by the addition of adenosine draws attention to the importance of the rNTP pools in viral replication. Finally, among the alphaviruses, SV_{PZF} is the only example so far of a drug-resistance mutation which maps to nsP4, the viral RDRP.

We isolated SV_{PZF} by selecting for a mutant able to replicate in PZF-treated mosquito cells. Thus the findings that SV_{PZF} was severely restricted in BHK cells and that this phenotype could be relieved by adenosine were surprising. We assumed initially that the restriction phenotype of SV_{PZF} could be explained by its altered RDRP. We postulated that perhaps the concentrations of UTP and CTP in BHK cells were too high for the optimal replication of SV_{PZF}, which we assumed encoded an RDRP adapted to low levels of these substrates. According to this hypothesis, the addition of adenosine to BHK cells by reducing the levels of UTP and CTP (Fig. 2B) would create an intracellular environment more favorable for the polymerase encoded by SV_{PZF}. This idea was also consistent with our observations that when infected mosquito cells were treated with low concentrations of PZF or PALA, both of which lower the levels of UTP and CTP (not shown), the yields of SV were increased up to 10-fold. However, the addition of PZF to BHK cells [PZF does not inhibit the replication of SV_{STD} in BHK cells (Lin *et al.,* 2000)] failed to relieve the restriction of SV_{PZF}. Furthermore, if the restriction were due to the mutant RDRP, one would expect that total viral RNA synthesis, both genomic and subgenomic, would be decreased in SV_{PZF}-infected BHK cells; our results, however, clearly show that the restriction phenotype was associated with a specific defect in the synthesis of SG RNA. Nevertheless, the results of experiments with the mutated double SG viruses showed that although the C7593A mutation at the −5 position of SG promoter 2 did reduce synthesis of SG RNA 2, both the P609T change in nsP4 and the mutation at the −5 position of SG promoter 2 were required to produce the full restriction phenotype of SV_{PZF} (Table 3).

That a reduction in the yield of Sindbis virus can result from mutations in the SG promoter and the resultant decreased synthesis of SG RNA has been convincingly documented by Hertz and Huang (1995a,b) in their work with double SG viruses. They noted, in fact, "a direct correlation between promoter activity and progeny virion production." Also noteworthy (in their studies they focused on the sequence of the SG promoter from positions −13 to −9) are their observations that deviations from the wild-type sequence in this region reduced the yield of progeny virus from mosquito cells much more drastically than from BHK cells. For example, their C3 virus, which had four changes from the -13 to -9 wild-type sequence, showed a 25-fold reduction in yield relative to the control in BHK cells, but a 2000-fold reduction in mosquito cells. We have not carried out extensive studies of our mutated double SG viruses in mosquito cells, but did observe that SV_{mSG2} replicated very poorly in mosquito cells. On the other hand, SV_{TOTO} containing only the C7593A mutation (mutation 4) replicated in mosquito cells as well as SV_{PZF}.

Turning to the question of how adenosine relieves the restriction of SV_{PZF} in BHK cells, we propose the following hypothesis to explain both the defect in SG RNA synthesis and the action of adenosine in reversing this phenotype. We suggest that the sequence change in the SG promoter, together with the amino acid changes in nsP4, reduces the affinity of the RNA polymerase for the SG promoter, which results in a destabilization of the RNA polymerase–promoter initiation complex and a decrease in the synthesis of SG RNA. Addition of adenosine to BHK cells dramatically increases the level of ATP (Fig. 1A). Since ATP is the initiating nucleotide for the synthesis of SG RNA, and thus part of the initiation complex, the increased concentration of ATP is able to compensate for the "destabilization" of the polymerase–promoter complex and subsequent weakening of the SG promoter activity (which result from the C7593A mutation), thus allowing the synthesis of normal amounts of SG RNA.

An attractive precedent for this hypothesis comes from studies on the *Escherichia coli* ribosomal RNA promoter, in which Gaal *et al.* (1997) showed that the sequence of this promoter influences not only the stability of the polymerase promoter complex, but also the concentration of the initiating rNTP required to initiate transcription. Thus in *in vitro* experiments, when a ribosomal RNA transcript began with an A, transcription was very sensitive to and dependent on the concentration of ATP, as opposed to the other rNTPs, but when it began with a G, it was very sensitive to the concentration of GTP. The presence of base substitutions or a deletion in the promoter sequence dramatically influenced both the stability of the polymerase–promoter complexes and the efficiency of transcription. These experiments demonstrated that the sequence of a promoter can determine the concentration of the initiating rNTP needed for maximal efficiency of transcription. We suggest that a similar situation exists with respect to the SV_{PZF} SG promoter, in that the transcription initiation complex, with the mutated SG promoter and altered nsP4, requires an elevated

concentration (above that normally present in the BHK cells) of the initiating nucleotide, ATP, to function efficiently.

We do note that the double SG virus with the SV_{PZF} 4 mutation only in SG promoter 1 showed a mild restriction in BHK cells (Table 3) even though it apparently made normal amounts of SG RNA 2 (Fig. 7) and the viral structural proteins (Fig. 6). At present, we have no explanation for why this is so.

Experiments are under way to see whether it is possible to change the initiating nucleotide of the SG transcript from an A to a G. If such a change is compatible with viability, we suggest that a C to A change at the −5 position of the SG promoter will still result in a restriction phenotype in BHK cells, but that the restriction would then be relieved most efficiently by addition of guanosine, rather than adenosine.

MATERIALS AND METHODS

Cells, viruses, and plasmids

Primary cultures of CEF, prepared as described previously (Stollar *et al.,* 1976), and BHK-21 cells (ATCC CCL10) were grown in Eagle's MEM supplemented with nonessential amino acids, 4 mM glutamine, 5% tryptose phosphate, and 5% bovine serum.

Our standard Sindbis virus has been described elsewhere (Shenk and Stollar, 1973). SV_{TOTO} is the virus derived from the plasmid pToto 1101 (Rice *et al.,* 1987). SV_{PZF} is a mutant of Sindbis virus resistant to PZF, a cytidine analogue (Lin *et al.,* 2000). Chimeric Sindbis viruses containing various sequences derived from the SV_{PZF} genome and viruses containing various combinations of the mutations found in SV_{PZF} were described by Lin *et al.* (2000).

BHK cells were infected with the indicated viruses at an m.o.i. of 10 PFU/cell (calculated by using the titers as determined on CEF) and maintained after infection at 34.5°C in medium containing 0.2% BSA in place of serum. Media from infected cultures were harvested 20 h after infection and virus yields were measured by plaque formation on primary cultures of CEF (Shenk *et al.,* 1974).

pTCS (14,473 bp, Fig. 5), which was kindly provided to us by Henry Huang, is a cDNA clone of Sindbis virus which contains two wild-type SG promoters. A bacterial chloramphenicol acetyltransferase cDNA is downstream of SG promoter 1 and the viral structural genes are downstream of SG promoter 2 (Hertz and Huang, 1995a,b).

Site-directed mutagenesis

To introduce the C7593A mutation of SV_{PZF} (Lin *et al.,* 2000) into the SG promoter 1 of pTCS, the *Spe*I–*Xho*I fragment (nt 5262–8317 of pTCS) containing the SG promoter 1 was subcloned into pSL1180 (Amersham Pharmacia Biotech). Site-directed mutagenesis was carried out using the QuikChange kit purchased from Stratagene. After mutagenesis, the presence of the desired mutation was confirmed by sequencing. The *Spe*I–*Xho*I fragment DNA sequence with the desired mutation was then exchanged back into pTCS and the presence of the mutation in pTCS was reconfirmed.

To introduce the C7593A mutation into the SG promoter 2, the *Xho*I–*Bss*HII (nt 8317–10,639) fragment was excised from pTCS and site-directed mutagenesis was performed as described above.

To generate pTCS with mutations in both subgenomic promoters, the mutated *Spe*I–*Xho*I and *Xho*I–*Bss*HII fragments just described were combined with the 9096-bp fragment from pTCS, which had been digested with *Spe*I and *Bss*HII (nt 5262/nt 10,639 in pTCS), and a three-fragment ligation was carried out. This gave a mutant pTCS containing C to A mutations at nt 7593 and 8422, respectively. The orientations and mutations in both promoters were confirmed by sequencing. DNA manipulations were performed by standard procedures (Sambrook *et al.,* 1989).

To generate virus with two SG promoters, wild-type or mutant pTCS was linearized with *Sst*I (nt 12,588 and 14,387 in pTCS) and infectious RNA transcripts were generated as described previously (Mi *et al.,* 1989). The RNA transcripts were transfected into primary cultures of CEF to generate infectious double SG viruses.

Synthesis of viral RNA and proteins in infected BHK cells

BHK cell cultures in 35-mm plates were infected with virus at an m.o.i. of 10 PFU/cell and maintained at 34.5° in medium containing 0.2% BSA, 0.5 μg actinomycin D/ml, and 300 μM adenosine as indicated.

To assess synthesis of viral RNA, cells were incubated with ^{32}P-labeled orthophosphate (100 μCi/35-mm plate) from 3 to 8 h after infection. The total RNA was isolated with TriZol reagent (Gibco BRL) and precipitated with isopropanol. Ten percent of the total RNA was denatured in buffer containing formaldehyde and de-ionized formamide and then resolved in 1% formaldehyde–agarose gels (Sambrook *et al.,* 1989). The gel was dried and exposed to X-ray film.

To assess synthesis of viral proteins, at the times indicated, medium was replaced with methionine-free medium, and then cells were labeled with ^{35}S-labeled protein labeling mix (NEN). Proteins in cell lysate corresponding to 0.5×10^6 cells were precipitated with acetone as described previously (Durbin and Stollar, 1984). The proteins were analyzed using 10% SDS–PAGE with a stacking gel. The gel was dried and exposed to X-ray film.

Extraction and assay of ribonucleotides

BHK cells were mock-infected and treated with adenosine as indicated; ribonucleotides were extracted from the cells as described by Bofill *et al.* (1995). Briefly, cells from each 35-mm plate were lysed with 200 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation for 1 min at 12,000*g*. The supernatant was back-extracted with diethyl ether to a pH of 5, and the ribonucleotide extracts were analyzed by HPLC (Fairbanks *et al.,* 1995).

ACKNOWLEDGMENTS

This work was supported by the US–Japan Medical Science Program through U.S. Public Health Service Grant AI-05920 from the National Institutes of Health and UMDNJ Foundation Grant 16-2000. We are also grateful to Lillian M. MacMath for her help in the preparation of the manuscript.

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